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# The cysteine proteinases of the pineapple plant

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The pineapple plant (*Ananas comosus*) was shown to contain at least four distinct cysteine proteinases, which were purified by a procedure involving active-site-directed affinity chromatography. The major proteinase present in extracts of plant stem was stem bromelain, whilst fruit bromelain was the major proteinase in the fruit. Two additional cysteine proteinases were detected only in the stem: these were ananain and a previously undescribed enzyme that we have called comosain. Stem bromelain, fruit bromelain and ananain were shown to be immunologically distinct. Enzymic characterization revealed differences in both substrate-specificities and inhibition profiles. A study of the cysteine proteinase derived from the related bromeliad *Bromelia pinguin* (pinguinain) indicated that in many respects it was similar to fruit bromelain, although it was found to be immunologically distinct.

## INTRODUCTION

Crude pineapple (*Ananas comosus*) stem preparations have previously been shown to contain two distinct cysteine proteinases, called stem bromelain and ananain (Rowan *et al.*, 1988). The major component, stem bromelain, has since been sequenced (Ritonja *et al.*, 1989) and shown to be a member of the papain superfamily. The major cysteine proteinase of pineapple fruit is called fruit bromelain, although both bromelain enzymes are included in EC 3.4.22.4 (IUB Enzyme Nomenclature Committee, 1984). There is considerable confusion as to whether these enzymes are distinct proteins (Ota *et al.*, 1972, 1985; Yamada *et al.*, 1976), or whether they represent two forms of a single enzyme (Iida *et al.*, 1973; Sasaki *et al.*, 1973). Heterogeneity of the proteinase population present in the juice of the pineapple fruit has long been suspected (Chittenden *et al.*, 1892; Bergmann *et al.*, 1937; Balls *et al.*, 1941). Several contradictory reports have described up to three different components (Ota, 1966; Ota *et al.*, 1961, 1964, 1972, 1985; Yamada *et al.*, 1976). Unlike crude stem bromelain, which is used widely in industry (for review see Caygill, 1979), fruit bromelain is not commercially available despite the large quantities of waste pineapple fruit portions at pineapple canneries (Caygill, 1979).

The fruits and stems of *Bromelia pinguin* (another member of the Bromeliaceae family) contain a proteinase called pinguinain (EC 3.4.99.18), which has been only partially characterized to date (Toro-Goyco *et al.*, 1968; Toro-Goyco & Rodriguez-Costas, 1976). However, the fruits of this plant (commonly called rat or mouse pineapple) have been reported to contain a proteolytic component that is immunologically related to fruit bromelain (Toro-Goyco & Rodriguez-Costas, 1976).

We have now examined an extract of pineapple stem and fresh pineapple fruit for the presence of cysteine proteinases. These have been purified and their immunological and enzymic properties compared with each other and with those of pinguinain.

## EXPERIMENTAL

### Materials

Many of the reagents (including affinity-purified ananain and stem bromelain) were obtained as described previously (Rowan *et al.*, 1988). In addition, fresh pineapple fruits were purchased from a local supermarket (country of origin Costa Rica). Pinguinain was from Sigma Chemical Co., Poole, Dorset, U.K. Bovine haemoglobin was prepared as previously described (Barrett, 1970). Chicken cystatin was a mixture of forms 1 and 2 purified by affinity chromatography (Anastasi *et al.*, 1983), titrated with papain that had previously been standardized by titration with compound E-64 (Zucker *et al.*, 1985). Bz-Phe-Val-Arg-NH-Mec was from Bachem Feinchemikalien, Bubendorf, Switzerland. Z-Phe-Cit-NH-Mec and Boc-Gly-NH-Mec were custom synthesized by Cambridge Research Biochemicals, Cambridge, U.K., and Z-Gly-Phe-Cit-NH-Mec was given by Dr. C. J. Gray, Department of Chemistry, University of Birmingham, Birmingham, U.K. Phe-GlySc was from Professor D. H. Rich, School of Pharmacy, University of Wisconsin–Madison, Madison, WI, U.S.A.

### Protein determination

Protein was quantified as absorbance units calculated as $A_{280,1cm}$ × volume (ml). $A^{1\%}_{280}$ values of 19.2, 20.1 and 24.6 were used for fruit bromelain, stem bromelain and pinguinain respectively (Yamada *et al.*, 1976; Murachi & Yasui, 1965; Toro-Goyco *et al.*, 1968).

### Enzyme assays

Stopped assays of the hydrolysis of peptidyl-NH-Mec derivatives, azocasein, azocoll, fibrin and hide powder

Abbreviations used: names of amino acids, peptides and their derivatives are abbreviated in accordance with the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (1984); additional abbreviations are: Ahx, 6-aminohexanoyl; compound E-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanidino)butane; GlySc, glycinaldehyde semicarbazone; -NH-Mec, 7-(4-methyl)coumarylamide; -NH-Np, *p*-nitroanilide.

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azure were as described previously (Rowan *et al.*, 1988). Activity against haemoglobin was determined by substituting this substrate in the azocasein protocol and measuring the $A_{280}$ of the supernatant.

Assays for the hydrolysis of peptidyl-NH-Np derivatives were performed as previously described (Buttle & Barrett, 1984) except that the final substrate concentration was 1.25 mM.

### Enzyme purification

**Comosain.** After cation-exchange chromatography on the Mono S f.p.l.c. column (Pharmacia, Uppsala, Sweden), activity against Z-Arg-Arg-NH-Mec was detected in the late-eluted ananain region (peak III in Fig. 1 of Rowan *et al.*, 1988). Comosain was affinity-purified from this late-eluted material essentially as described for ananain (Rowan *et al.*, 1988), except that the ligand used was Phe-GlySc instead of Gly-Phe-GlySc. Enzymic activities were monitored with Z-Arg-Arg-NH-Mec for comosain and Z-Phe-Arg-NH-Mec for ananain. The material eluted by disulphide from the Sepharose–Ahx-Phe-GlySc column was further subjected to three stages of f.p.l.c. on the Mono S column at pH 9.0 as described previously for ananain (Rowan *et al.*, 1988), except that fractions containing activity against Z-Arg-Arg-NH-Mec (preceding the major protein peak, which was associated with Z-Phe-Arg-NH-Mec-hydrolysing activity) were combined, diluted with 2 vol. of 25 mM-sodium tetraborate/1 mM-EDTA, pH 9.0, and re-run on the same Mono S column. After the third stage of cation-exchange chromatography, the fraction with the highest specific activity against Z-Arg-Arg-NH-Mec was dialysed into 1 mM-EDTA/0.05% Brij 35 and kept at 4 °C.

**Fruit bromelain.** Fruit bromelain was purified from fresh pineapple fruit. The leaves and outer husk were removed from the fruit, and the main fruit portion (850 g) was diced into small cubes and minced in a blender. This was then mixed with 500 ml of 1 mM-EDTA and filtered, under vacuum, through Whatman type 540 paper. The resulting extract (930 ml) was acetone-fractionated by the method of Ota *et al.* (1964), modified for the use of a filter aid (Hyflo Super-Cel) following the method of Barrett (1970). Two precipitates were thus obtained, P1 being insoluble in 50% (v/v) acetone, and P2 in 66% (v/v) acetone. The P2 fraction was extracted from the filter cake by three treatments with 5 mM-EDTA (10 ml/100 ml of extract, for each treatment), which were combined and kept at 4 °C.

The material from the first acetone precipitation (P1) was dissolved in 100 ml of 5 mM-EDTA containing 0.1% (w/v) Brij 35. Samples of both fraction P1 and fraction P2 were clarified and chromatographed separately on the Mono S column at pH 5.0 essentially as described previously for crude pineapple stem material (Rowan *et al.*, 1988), except that the buffers contained 0.1% Brij 35.

A sample (approx. 20 mg) of the major protein peak after chromatography of the P2 acetone fraction was affinity-purified on a column of Sepharose–Ahx-Phe-GlySc essentially as described for ananain (Rowan *et al.*, 1988), except that continuous elution from the affinity column was performed with 50 mM-sodium acetate buffer in water/ethanediol (2:1, v/v), pH 4.5, containing 10 mM-$HgCl_2$. The $HgCl_2$-eluted material was subjected to two stages of cation-exchange chromatography on the Mono S column at pH 5.0 as described previously (Rowan *et al.*, 1988). The fractions active against Z-Phe-Arg-NH-Mec were combined and dialysed against 1 mM-EDTA before being freeze-dried.

**Pinguinain.** Commercial pinguinain (approx. 2 mg) was dissolved in 10 ml of 1 mM-EDTA, and chromatographed on the Mono S column at pH 5.0 as described previously for crude pineapple stem extract (Rowan *et al.*, 1988). Pinguinain (active against Bz-Phe-Val-Arg-NH-Mec) thus obtained was further purified from the major protein peak by affinity chromatography, exactly as described for fruit bromelain.

### Gel electrophoresis

SDS/PAGE was performed, with reduction, in slab gels containing 12.5% (w/v) total acrylamide as described by Bury (1981).

Multizonal cathodal electrophoresis employing the buffer system of Thomas & Hodes (1981) and 12.5% (w/v) acrylamide was otherwise as described by Zucker *et al.* (1985).

### Immunological methods

The antisera to crude Sigma pineapple stem extract and ananain were those described previously (Rowan *et al.*, 1988). Antisera were also raised against affinity-purified preparations of stem bromelain and fruit bromelain that had been mildly carboxymethylated (Zucker *et al.*, 1985) before injection. The antisera were raised in rabbits by a single intramuscular injection of 100 $\mu$g of antigen in Freund's complete adjuvant, followed by two subcutaneous injections of 50 $\mu$g in incomplete adjuvant at fortnightly intervals.

IgG was partially purified from the antisera by $(NH_4)_2SO_4$ fractionation (Heide & Schwick, 1978), followed by dialysis into 0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4.

Double-immunodiffusion analysis was done as described previously (Buttle & Barrett, 1984).

### Active-site titration

The pineapple proteinases were titrated with compound E-64 as previously described (Rowan *et al.*, 1988).

### pH optima of the pineapple proteinases

The rate of azocasein hydrolysis was examined over the pH range 4.0–9.0 as described previously by Buttle *et al.* (1989), except that 4 mM-cysteine was used as the activator.

### Determination of kinetic parameters

The kinetic parameters for the hydrolysis of coumarylamide substrates were determined by using previously described methods (Rowan *et al.*, 1988), with 4 mM-cysteine as activator.

### Inhibition by compound E-64 and chicken cystatin

The second-order rate constants, $k_2$, for the inactivation of fruit bromelain and pinguinain by compound E-64, and the inhibition constant, $K_i$, for inhibition by chicken cystatin, were investigated exactly as described previously (Rowan *et al.*, 1988), except that Bz-Phe-Val-Arg-NH-Mec was used as substrate (0.5 $\mu$M final concentration) and 4 mM-cysteine as activator. Fruit bromelain (20 pM final active concentration) or

pinguinain (30 pM) was incubated with chicken cystatin in the concentration range 0.0005–1.12 $\mu$M, or with compound E-64 in the concentration range 0.25–1.50 $\mu$M. Values of $k_2$ were found by the equation:

$$k_2 = k_{2(app.)} \cdot (1 + [S]/K_m)$$

## RESULTS AND DISCUSSION

### Purification

**Comosain.** Activity against Z-Arg-Arg-NH-Mec was detected in the late-eluted region (peak III in Fig. 1 of Rowan *et al.*, 1988) after chromatography on the Mono S column at pH 5.0. Since this substrate is only poorly hydrolysed by the major protein in this peak (ananain) (Rowan *et al.*, 1988), the activity was attributed to another proteinase, which was designated comosain.

The purification of comosain was made difficult by the presence of much greater amounts of ananain in the material applied to the affinity column. Comosain and ananain were eluted together from the affinity column, and the subsequent separation of these two proteinases was dependent on repeated partial separation on the Mono S column. Finally, a single peak of activity against Z-Arg-Arg-NH-Mec was obtained (Fig. 1). This fraction was subsequently shown still to contain some activity against Z-Phe-Arg-NH-Mec and Bz-Phe-Val-Arg-NH-Mec, consistent with trace contamination by ananain.

**Fruit bromelain.** The initial acetone precipitation of crude pineapple fruit juice resulted in two protein fractions (P1 and P2). Chromatography on the Mono S column at pH 5.0 of the material from the P1 fraction resolved two protein peaks (I and II) possessing different substrate-specificities (see Fig. 2*a*). The second acetone fraction (P2) yielded a partially resolved double peak (Fig. 2*b*), which corresponded in elution position to the first peak (peak I in Fig. 2*a*) of the P1 fraction. Since this was the major protein component of the pineapple fruit juice, it was designated fruit bromelain. Fruit bromelain has been purified previously from such an acetone fraction (Ota *et al.*, 1964).

Fruit bromelain was further purified from this major protein peak (Fig. 2*b*) on a column if Sepharose–Ahx-Phe-GlySc. Elution with $HgCl_2$ resulted in over 90% recovery of the activity applied, and after two further stages of cation-exchange chromatography (not shown) this material was found to be 85% active cysteine proteinase by active-site titration.

**Pinguinain.** Cation-exchange chromatography of commercial pinguinain yielded a major single protein peak, which was eluted in a similar position to fruit bromelain (see Fig. 3), although other minor peaks were also evident. The major peak (marked by the bar in Fig. 3) was designated pinguinain, and after affinity chromatography was found to be 80% active cysteine proteinase by titration with compound E-64.

### Electrophoretic profiles

The apparent $M_r$ of fruit bromelain was 25000 (Fig. 4*a*). This is very similar to that of ananain, but less than that of stem bromelain in this gel system (Rowan *et al.*, 1988). Pinguinain had an apparent $M_r$ value of about 26000 (not shown).

Because of the acidic nature of fruit bromelain (Yamada *et al.*, 1976), it failed to enter the multizonal cathodal gel (see Fig. 4*b*). The late-eluted material in the P1 acetone fraction from the fruit (peak II in Fig. 2*a*) resembled stem bromelain purified from pineapple stem in its charge, although microheterogeneities did exist (Fig. 4*b*). No ananain was detected in the fruit fractions.

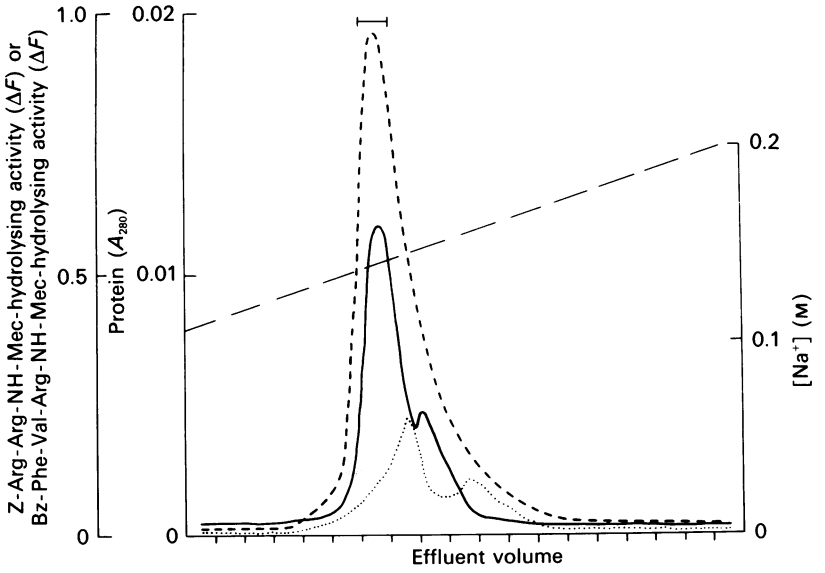


**Fig. 1. Cation-exchange chromatography of comosain on the Mono S HR5/5 column**

The material eluted from the Sepharose–Ahx-Phe-GlySc column was applied to, and eluted from, the cation-exchange column three times, the last of which resulted in the chromatograph shown. The material marked by the bar was designated comosain, and was used for further characterization. See the Experimental section for details. ——, Protein ($A_{280}$); – – – –, Z-Arg-Arg-NH-Mec-hydrolysing activity; ·····, Bz-Phe-Val-Arg-NH-Mec-hydrolysing activity; ——, counterion ($Na^+$) concentration.

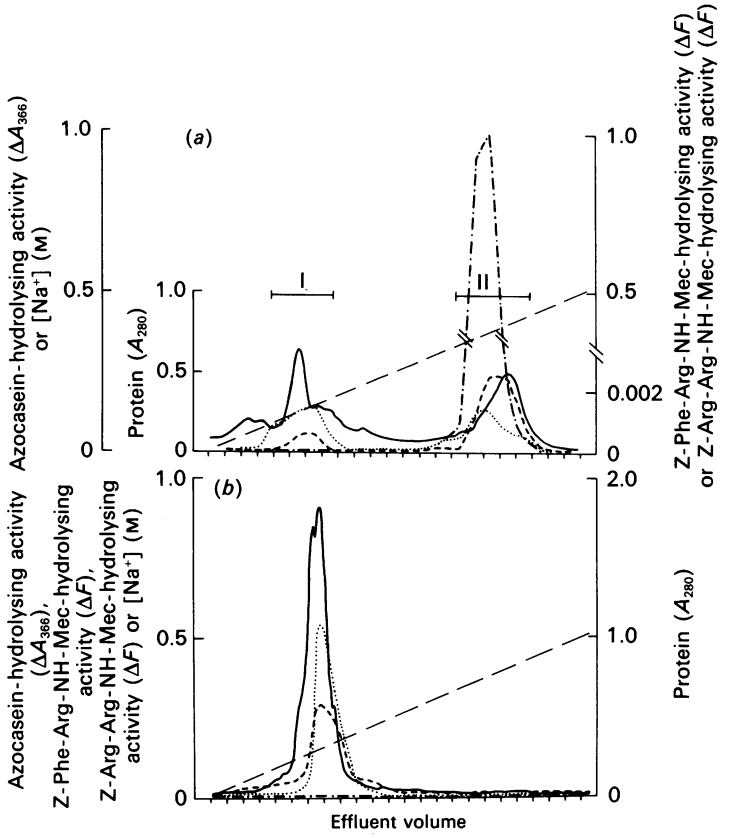


**Fig. 2. Cation-exchange chromatography at pH 5.0 of the acetone fractions obtained from a pineapple fruit extract**

(*a*) Fraction P1; (*b*) fraction P2. ——, Protein ($A_{280}$); ·····, Z-Phe-Arg-NH-Mec-hydrolysing activity; – – – –, azocasein-hydrolysing activity; ·–·–·, Z-Arg-Arg-NH-Mec-hydrolysing activity; ——, counterion ($Na^+$) concentration.

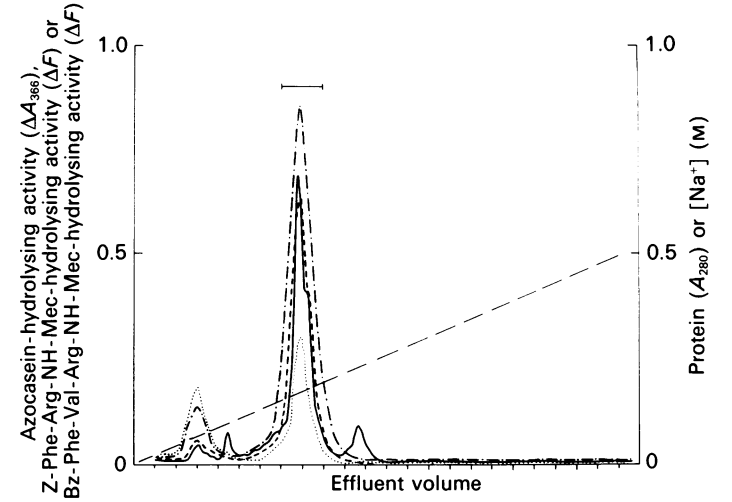


**Fig. 3. Cation-exchange chromatography at pH 5.0 of commercial pinguinain**

Commercial pinguinain (approx. 1 mg) was applied to the Mono S HR5/5 column and eluted as described in the Experimental section. The fractions (0.5 ml) marked by the bar were combined and used subsequently for affinity purification of pinguinain. ——, Protein ($A_{280}$); ·····, Z-Phe-Arg-NH-Mec-hydrolysing activity; ·–·–·, Bz-Phe-Val-Arg-NH-Mec-hydrolysing activity; – – – –, azocasein-hydrolysing activity; ——, counterion ($Na^+$) concentration.

## Double immunodiffusion

Fruit bromelain was shown to be totally distinct from stem bromelain, although, as predicted from the experiments described above, both bromelain enzymes were indeed present in the fruit (Fig. 5). Since one of the antisera used was a multivalent antiserum raised against all the pineapple stem proteins, it can be concluded that both these enzymes are also present in the pineapple stem. This finding explains the previous reports of cross-

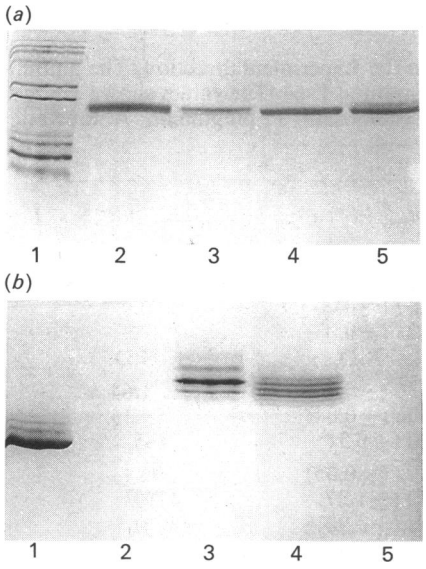


**Fig. 4. Gel electrophoresis of the pineapple proteinases**

(*a*) SDS/PAGE with reduction of: lane 1, $M_r$ standards; lane 2, peak II in Fig. 2(*a*); lane 3, stem bromelain; lane 4, fruit bromelain; lane 5, crude pineapple fruit extract. (*b*) Multizonal cathodal gel electrophoresis of: lane 1, ananain; lane 2, fruit bromelain; lane 3, stem bromelain; lane 4, peak II in Fig. 2(*a*); lane 5, crude pineapple fruit extract.

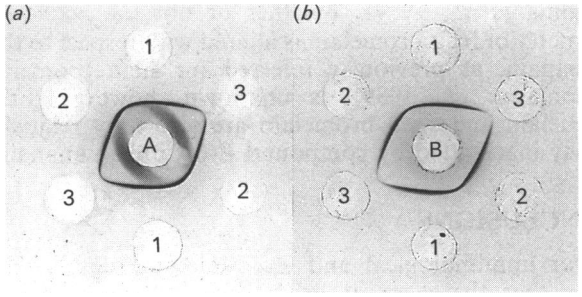


**Fig. 5. Double immunodiffusion of fruit bromelain**

The wells contained: A, anti-(pineapple stem protein) serum; B, anti-(stem bromelain) IgG; 1, peak II in Fig. 2(*a*); 2, stem bromelain; 3, fruit bromelain.

reaction between stem bromelain and fruit bromelain (Sasaki *et al.*, 1973; Iida *et al.*, 1973). No reactions were detected between fruit bromelain and anti-ananain serum (not shown).

Double-immunodiffusion analysis of pinguinain against anti-(fruit bromelain) and anti-(stem bromelain) IgG preparations, and against anti-ananain serum, failed to detect any immunological cross-reaction between pinguinain and the pineapple proteinases (not shown).

### Catalytic activities of the pineapple proteinases

The specific activities of fruit bromelain and ananain were compared with those of stem bromelain with a variety of protein substrates at pH 6.8 (the pH optima for these proteinases were all quite broad and close to neutral pH; Rowan, 1989). Ananain and fruit bromelain had markedly higher activities than stem bromelain against all the substrates tested (Table 1).

Marked differences in specificity between the pineapple proteinases were seen with peptidyl-NH-Mec substrates (Table 2). Z-Phe-Arg-NH-Mec and Z-Arg-Arg-NH-Mec were previously shown to be good substrates for ananain and stem bromelain respectively (Rowan *et al.*, 1988), but a larger series of peptidyl-NH-Mec substrates had now been used. In view of the broad specificity of stem bromelain for proteins implied by the formation of small peptides for azocasein, fibrin and phosphorylase *a* (Table 1; Rowan *et al.*, 1988), it was surprising that stem bromelain only hydrolysed the dibasic substrate Z-Arg-Arg-NH-Mec (Table 2). The value of the Michaelis constant, $K_m$, of comosain for Z-Arg-Arg-NH-Mec clearly shows that this proteinase is distinct from any of the other pineapple enzymes (Table 2). Both ananain and fruit bromelain had broad specificities, with Bz-Phe-Val-Arg-NH-Mec being particularly susceptible. Pinguinain was found to have very similar kinetic constants to fruit bromelain with this substrate (Table 2).

For all the pineapple proteinases, no detectable amounts of aminomethylcoumarin were released from any of the following substrates: Gly-NH-Mec, Boc-Gly-NH-Mec, Ac-Lys-NH-Mec, Boc-Glu-Lys-Lys-NH-Mec, Z-Arg-NH-Mec, Gly-Gly-Arg-NH-Mec, Boc-Val-Pro-Arg-NH-Mec, Glt-Gly-Gly-Phe-NH-Mec and Suc-Ala-Ala-Pro-Phe-NH-Mec.

Relative rates of hydrolysis of Z-Arg-Arg-NH-Np, Z-Phe-Arg-NH-Np and Bz-Phe-Val-Arg-NH-Np suggested that the enzymes show similar specificities for nitroanilide (not shown) and aminomethylcoumarylamide substrates.

**Table 1. Relative activities of the pineapple proteinases against several protein substrates**

The assays were performed with equimolar amounts of active enzyme as described in the Experimental section. Arbitrary enzyme units were normalized to the protein-degrading activity of stem bromelain.

| | Specific activity relative to stem bromelain | | |
|---|---|---|---|
| Substrate | Stem bromelain | Fruit bromelain | Ananain |
| Azocasein | (1.00) | 1.49 | 1.90 |
| Hide powder | (1.00) | 1.71 | 2.01 |
| Azocoll | (1.00) | 4.02 | 3.14 |
| Haemoglobin | (1.00) | 1.50 | 4.59 |
| Fibrin | (1.00) | 2.67 | 5.36 |

**Table 2. Kinetic parameters for the hydrolysis of some peptidyl-NH-Mec substrates by the pineapple proteinases**

Continuous rate assays with peptidyl-NH-Mec substrates were as described in the Experimental section. The amount of each proteinase (except comosain) was standardized by active-site titration with Compound E-64. The values shown are means ± S.D. Key to proteinases: SB, stem bromelain; FB, fruit bromelain; A, ananain; C, comosain; P, pinguinain. Abbreviation: N.D., not determined.

| Substrate | Proteinase | $K_m$ ($\mu$M) | $k_{cat.}$ ($s^{-1}$) | $k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$) |
|---|---|---|---|---|
| Z-Arg-Arg-NH-Mec | SB | 15.4 (±2.8) | 27.26 (±1.88) | 1770 |
| | FB | 89.1 (±9.6) | 0.002 (±0.0001) | 0.02 |
| | A | 44.5 (±9.0) | 0.321 (±0.03) | 7.21 |
| | C | 1.4 (±0.1) | N.D. | N.D. |
| Z-Phe-Arg-NH-Mec | SB | 83.1 (±14.2) | 0.136 (±0.01) | 1.64 |
| | FB | 33.5 (±11.9) | 0.306 (±0.04) | 9.13 |
| | A | 48.4 (±5.3) | 7.00 (±0.34) | 145 |
| Bz-Phe-Val-Arg-NH-Mec | SB | 9.7 (±1.5) | 0.893 (±0.05) | 92.1 |
| | FB | 4.0 (±0.9) | 18.82 (±1.27) | 4710 |
| | A | 13.1 (±2.1) | 63.78 (±3.94) | 4870 |
| | P | 2.7 (±0.3) | 15.50 (±0.48) | 5740 |
| Z-Gly-Phe-Cit-NH-Mec* | SB | 15.3 (±3.3) | 0.12 (±0.02) | 7.84 |
| | FB | 18.7 (±15.7) | 0.38 (±0.39) | 20.3 |
| | A | 4.4 (±1.3) | 3.54 (±0.60) | 805 |
| Z-Phe-Cit-NH-Mec* | A | 11.1 (±4.2) | 20.96 (±6.31) | 1890 |

* The data for these substrates should be regarded as estimates only, owing to their poor solubility; substrate was used in the range 1–5 $\mu$M.

Bz-Arg-NH-Np was not significantly hydrolysed by any of the pineapple proteinases, contrary to previous reports (e.g. Ota *et al.*, 1964).

### Kinetic constants of inhibition

The $K_i$ values for inhibition of the pineapple proteinases by chicken cystatin and the $k_2$ values for inactivation by compound E-64 are presented in Table 3. No inhibition by chicken cystatin of fruit bromelain was detected, even in the presence of 1.12 $\mu$M inhibitor. In this respect fruit bromelain resembles stem bromelain; the two bromelain enzymes are unusual among cysteine proteinases of the papain superfamily in their resistance to inhibition by chicken cystatin, papaya proteinase IV being the only other known example (Buttle *et al.*, 1989). In view of this, it is noteworthy that pinguinain, which appears to be very similar to fruit bromelain in substrate-specificity (Table 2), is nonetheless strongly inhibited by chicken cystatin. This lends further support to our suggestion that substrate-specificity alone cannot explain susceptibility to inhibition by this class of inhibitor (Ritonja *et al.*, 1989). Whether or not the active-site geometry of fruit bromelain is altered with respect to that of papain, as previously inferred for stem bromelain (Ritonja *et al.*, 1989), is unknown, however. Fruit bromelain and stem bromelain are also only relatively slowly inactivated by compound E-64, unlike ananain.

**Table 3. Inhibition constants of the pineapple proteinases**

The values for kinetic constants of inhibition were determined by using continuous rate assays at pH 6.8 at 40 °C.

| | Compound E-64 $k_2$ ($M^{-1} \cdot s^{-1}$) | Chicken cystatin $K_i$ (M) |
|---|---|---|
| Ananain | 302297*† | $1.1 \times 10^{-9}$* |
| Stem bromelain | 678*† | $3.6 \times 10^{-5}$* |
| Fruit bromelain | 3385 | $> 1.1 \times 10^{-6}$ |
| Pinguinain | 9912 | $1.6 \times 10^{-9}$ |

* From Rowan *et al.* (1988).
† $k_2$ was calculated from $k_{2(app.)}$ as described in the Experimental section.

## CONCLUSIONS

Our immunological and enzymological data clearly show that there are at least three and perhaps four distinct cysteine proteinases present in crude pineapple stem extracts: stem bromelain (the major component), ananain, comosain and fruit bromelain. Pineapple fruits contain two of these enzymes: the major component is fruit bromelain, with stem bromelain being present in much smaller amounts.

The name 'bromelin' was first applied to the fruit enzyme (Chittenden *et al.*, 1892). Later, the term 'bromelain' was introduced and originally applied to 'any protease from any plant member of the plant family Bromeliaceae' (Heinicke, 1953). In view of the diverse properties of the various cysteine proteinases present in the plants of this family, such a broad term now has little value. However, with respect to the pineapple plant (*Ananas comosus*) it would seem pertinent to continue with the use of the trivial names stem bromelain and fruit bromelain until separate EC numbers are allocated to them.

Pinguinain (EC 3.4.99.18) is currently classified in the category of unknown catalytic mechanism. The results with the class-specific inhibitors of cysteine proteinases, chicken cystatin and compound E-64, clearly show that this enzyme is a cysteine proteinase, most probably of the

papain superfamily, and should now be classified as such.

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